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Coordinate Expression of Amplified Metallothionein I and II Genes in Cadmium-Resistant Chinese Hamster Cells

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Recombinant DNA probes complementary to Chinese hamster metallothionein (MT)-1 and MT-2 mRNAs were used to compare MT gene copy numbers, zinc-induced MT mRNA levels, and uninduced MT mRNA levels in cadmium-resistant (Cd^r) Chinese hamster ovary cell lines. Quantitative hybridization analyses determined that the MT-1 and MT-2 genes are each present at approximately single-copy levels in the genome of cell line Cd^r2C10 and are coordinately amplified approximately 7, 3, and 12 times over the Cd^r2C10 value in the genomes of cell lines Cd^r20F4, Cd^r30F9, and Cd^r200T1, respectively. The maximum zinc-induced MT-1 mRNA concentrations in cell lines Cd^r20F4, Cd^r30F9, and Cd^r200T1 were equal to 1, 3, and 15 times that measured in Cd^r2C10, respectively. Similarly, the maximum zinc-induced MT-2 mRNA concentrations were equal to 1, 3, and 14 times that measured in Cd^r2C10, respectively, and in each instance they were 90 to 150 times greater than their respective concentrations in uninduced cells. Thus, relative MT gene numbers are closely correlated with both zinc-induced and uninduced MT mRNA levels in Cd^r2C10, Cd^r30F9, and Cd^r200T1, but not in Cd^r20F4. Each of the latter two lines possesses structurally altered chromosomes whose breakpoints are near the MT locus. Nonetheless, the ratio of the levels of MT-1 to MT-2 mRNAs was constant in each of the four cell lines, including Cd^r20F4. These results demonstrate that MT-1 and MT-2 mRNAs are induced coordinately in each Cd^r cell line. Therefore, the coordination of the induction of MT-1 and MT-2 mRNA is independent of MT gene amplification, MT gene rearrangement, and the relative inducibilities of amplified MT genes. However, MT mRNA and protein levels each indicate that MT-1 and MT-2 expression is non-coordinate in uninduced cells. Thus, regulation of MT expression may involve two different mechanisms which are differentially operative in induced and uninduced cells.

The genomes of mammals encode two closely related metallothioneins (MTs), MT-1 and MT-2, whose expression is induced coordinately by either metals or glucocorticoids (1, 13, 17). Two different mechanisms have been advanced to account for the coordinate induction of mammalian MT genes. The first has been proposed to explain human MT regulation and is based on differences in the gene dosage and relative inducibilities of the human MT-1 and MT-2 genes. In humans, the MT gene cluster is composed of several functional MT genes and nonfunctional pseudogenes which are located at a number of different chromosomal loci (12, 18, 19). Mutagenesis and gene transfer experiments have identified regulatory regions upstream from each human MT gene promoter. The sequences upstream from the MT-2 promoter mediate transcription induction by zinc, cadmium, and glucocorticoids (12). In contrast, the sequences upstream from the MT-1 promoter mediate transcription induction by cadmium but are much less responsive to zinc and glucocorticoids (18). Nonetheless, since there are more MT-1 than MT-2 genes, both MT species can be induced coordinately by zinc and glucocorticoids (18).

A second mechanism has been proposed to explain mouse MT regulation and is based on the equivalence of the regulatory elements of the two murine MT genes. Unlike the human MT gene cluster, murine MT genes are present at single-copy levels and are closely linked at a single site in the genome (3, 20). Yagle and Palmiter (25) have reported that MT-1 and MT-2 genes respond identically to zinc, cadmium, copper, and glucocorticoids. The regulatory sequences upstream from the MT-1 and MT-2 genes are the binding sites for a *trans*-acting factor (21) that regulates metal-induced transcription from each downstream promoter (9, 16). They argue that if the upstream sequences of the two MT genes are functionally equivalent, then transcription of the two MT genes will be regulated coordinately (25). However, Crawford et al. (4) have shown that uninduced Chinese hamster cells synthesize MT-2 but not MT-1, although both MTs are coordinately induced when the cells are incubated with either zinc or cadmium. If the regulatory elements of the two MT genes are functionally equivalent, then the ratio of MT-1 to MT-2 expressed in induced and uninduced cells should be identical. The fact that they are not suggests that the MT genes can also be regulated independently.

Additional regulatory elements may also be involved in controlling MT gene expression in rodents. For example, the regulatory sequences that mediate metal-induced transcription are not alone sufficient for glucocorticoid-induced transcription (9, 16). Likewise, amplification of murine MT genes can cause the loss of glucocorticoid inducibility without affecting metal inducibility (15). Since glucocorticoids induce both murine MTs coordinately (25), these results could indicate that a single *cis*-acting element controls the glucocorticoid inducibility of pairs of linked MT-1 and MT-2 genes (16).

One means of evaluating these possibilities is to compare the extent and coordination of induced and constitutive MT gene expression in rodent cells possessing structural or numerical anomalies or both of the MT gene loci. If each MT gene is regulated independently, then rearrangements that affect the induction of one MT gene need not affect the

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induction of the other. However, if coordinate induction requires the action of a single *cis*-acting sequence, then either the rearrangement or underamplification of this sequence would be expected to influence coordinately the inducibility of both genes. This approach may also resolve DNA sequences necessary for MT gene induction from those required for MT gene transcription. Sequences required only for induction would not be expected to affect constitutive levels of MT expression in uninduced cells. In contrast, sequences required for the transcription of one, the other, or both MT genes would affect their expression in both induced and uninduced cells. Such studies would define more completely the differences and similiarities between MT regulation in rodents and primates.

Several cadmium-resistant (Cd^r) Chinese hamster cell lines have been derived and characterized, which provide a unique experimental system with which to study the regulation of the MT genes of rodents. These Cd^r lines each differ in their extents of MT inducibility (4, 5, 8, 11, 23) and in their MT gene dosage (4). Some of these lines also carry independent chromosome translocations whose breakpoints are near the amplified MT gene domains (4, 22). Previous studies of these translocations support the conclusion that these rearrangements occurred before the amplification of the MT genes (22). Thus, any changes in the MT genes or their linked regulatory sequences that resulted from these rearrangements would be expected to be present in each member of the amplified MT array. The results of investigations of the effects of these numerical and structural aberrations on the extent and coordination of MT gene expression are reported herein. These results are consistent with the conclusion that the expression of the MT genes is regulated by two different mechanisms which are differentially operative in induced and uninduced cells.

MATERIALS AND METHODS

Nucleic acid isolation. (i) DNA. Chinese hamster cell nuclei were isolated by previously described techniques and lysed with sodium dodecyl sulfate (SDS) and proteinase K (Beckman Instruments, Inc., Fullerton, Calif.) (4). The mixture was then extracted with phenol-chloroform, and DNA was precipitated with ethanol. After suspension, DNA to be used in solution hybridization was fragmented by high-speed homogenization to an average single-strand length of 500 nucleotides (NT) (2). At least three independent preparations of the DNA of each cell line were purified over a 24-month period and used interchangeably throughout this study.

Recombinant MT DNA was purified from *Escherichia coli* HB101 strains harboring plasmids pCHMT-1 or pCHMT-2 (7) by standard procedures (14). Purified plasmid DNA was digested with the specified restriction enzymes as directed by the suppliers and, where indicated, resolved according to size in agarose gels. The indicated restriction fragments were then recovered from the gel by electroelution.

(ii) RNA. The four Cd^r Chinese hamster cell lines were grown in Spinner flask culture as previously described (11). To induce MT mRNA, zinc chloride was added to the cultures to a final concentration of 100 μM. To monitor the kinetics of MT mRNA induction, samples of cells were removed at various times after the addition of the $ZnCl_2$ and pulse-labeled for 30 min with [^{35}S]cysteine. Cytoplasmic extracts containing the pulse-labeled proteins were then resolved by Sephadex G-75 chromatography, and the ratio of MT to non-MT protein radioactivity was computed at each time point (see Table 3) as described previously (10, 11). This ratio is assumed to reflect the relative amounts of MT and non-MT mRNAs present on the polysomes at the time of the assay. The validity of this approach was established previously by Hildebrand et al. (10). These authors compared the kinetics of MT mRNA induction defined by the ratio of the pulse-labeled MT and non-MT proteins to that determined from the amounts of MT protein synthesized in a cell-free translation system programmed with poly(A)$^+$ RNA isolated from parallel samples of cells. (No MT protein synthesis is directed by poly(A)$^-$ RNA.) Both methods defined almost identical MT mRNA induction kinetics. By these criteria, maximal MT mRNA levels were induced in the present study in Cd^r2C10, Cd^r20F4, Cd^r30F9, and Cd^r200T1 cells after exposures to 100 μM $ZnCl_2$ of approximately 8 to 10, 8, 6, and 2 h, respectively (see Table 3). Cytoplasmic RNA was isolated from paired 2-liter cultures of uninduced and zinc-induced cells, purified with phenol-chloroform, and precipitated with alcohol, and the poly(A)$^+$ fraction was purified by chromatography on oligo(dT) cellulose, each as previously described (8). Purified RNA was dissolved in water and stored frozen at −20°C before use in molecular hybridization analyses.

DNA probe preparation. ^{3}H-labeled genomic DNA was prepared by nick translation (14), and the single-copy DNA fraction was purified by kinetic fractionation. Briefly, nick-translated genomic DNA was allowed to self-reassociate at 72°C in 1.5 M NaCl–10 mM Tris hydrochloride (pH 7.5)–2 mM tetrasodium EDTA–0.2% SDS to an equivalent C_0t (i.e., DNA concentration × time × salt rate factor [reference 2]) of 10^3, and the single- and double-strand DNA fractions were separated by hydroxylapatite chromatography. ^{3}H-labeled cDNA complementary to metal-induced mRNA was prepared as previously described (8).

^{32}P-labeled MT DNA was synthesized by T4 polymerase substitution or reverse transcription end labeling (14) of either a 160-NT *Bam*HI-*Hin*fI restriction fragment or a 63-NT *Hpa*II-*Alu*I restriction fragment carried by the cDNA insert of plasmid pCHMT-2 (7). Each of these fragments defines the translated portion of MT-2 mRNA. They share 78 and 84% nucleotide sequence homology, respectively, with the comparable translated portion of MT-1 mRNA and hybridize equally in Southern blot analysis to both MT-1 and MT-2 genes (4, 7).

^{32}P-labeled DNA containing sequences specific for MT-1 or MT-2 mRNA was prepared from the *Bgl*II-*Hpa*II restriction fragment of the cDNA insert carried by plasmid pCHMT-1 and the *Bam*HI-*Hin*fI fragment carried by plasmid pCHMT-2, respectively. Approximately 100 ng of each ^{32}P-labeled fragment was mixed with approximately 10 μg of poly(A)$^+$ mRNA from maximally zinc-induced Cd^r200T1 Chinese hamster cells in 300 μl of hybridization buffer (1.5 NaCl, 10 mM Tris hydrochloride [pH 7.5], 2 mM tetrasodium EDTA, 0.2% SDS). The samples were melted at 100°C for 2 min and reannealed at 72°C to an equivalent R_0t of 0.5 to hybridize MT mRNA and the complementary strand of labeled plasmid DNA. Nonhybridized, single-stranded RNA and plasmid DNA molecules were hydrolyzed with S1 nuclease in 15 ml of S1 nuclease buffer (8) at 37°C for 60 min. Approximately 70% of the ^{32}P-labeled DNA was S1 nuclease resistant. Tetrasodium EDTA was then added to 10 mM, Tris hydrochloride (pH 7.5) to 10 mM, SDS to 0.1%, tRNA carrier to 70 μg/ml, and sodium acetate to 300 mM. The mixture was then extracted with phenol-chloroform and precipitated with three volumes of ethanol. The sample was then dissolved in 0.5 N NaOH and incu-

bated at 37°C for 18 h to hydrolyze RNA. The resulting single-stranded DNA probes were then neutralized, dialyzed, precipitated with ethanol and tRNA carrier, dissolved in water, and stored frozen at −20°C. The final yield was approximately 25% of the initial ^{32}P-labeled DNA.

Single-stranded cDNA complementary to abundant, metal-induced RNA was prepared as described by Griffith et al. (8). Greater than 60% of this tracer was recovered in DNA duplexes after hybridization with a mixture of cloned pCHMT-1 and pCHMT-2 DNAs.

Measurement of metallothionein gene copy number. (i) Renaturation kinetics. Radiolabeled MT and genomic DNA tracers were prepared as described above and mixed with sequence excesses of unlabeled, 500-NT total genomic DNA from each Cd^r cell line in 10 to 50 μl of 1.5 M NaCl–10 mM Tris hydrochloride (pH 7.5)–2 mM tetrasodium EDTA–0.2% SDS. The samples were denatured by heating for 2 min at 100°C and incubated at 72°C until the desired equivalent C_0t was achieved. The samples were then diluted with 1 ml of 0.05 M sodium phosphate buffer (pH 7.0) and loaded onto 1-g hydroxylapatite columns at 60°C, and the single- and double-strand fractions were eluted with 0.12 and 0.4 M sodium phosphate buffer (pH 7.0), respectively (2).

(ii) DNA dot-blot analysis. Dilutions of DNA of each Cd^r cell line were suspended in 10 mM Tris hydrochloride (pH 7.5)–1.0 mM tetrasodium EDTA and denatured by addition of 0.1 volume of 3.0 M NaOH and incubation for 0.5 to 1.0 h at 60 to 70°C. After cooling to room temperature, samples were neutralized with 1.0 volume of 2.0 M ammonium acetate (pH 7.0). Samples were then dot-blotted onto BA85 nitrocellulose with a Minifold I (Schleicher & Schuell, Inc., Keene, N.H.) according to instructions provided by the manufacturer. Prehybridization and hybridization conditions were as described previously for Southern blot filter hybridizations (4). Various autoradiographic exposures (24 to 72 h) permitted evaluation of gene copy number by comparison of appropriate dilutions of the DNAs of Cd^r variant cells with known standards. Additionally, control experiments (data not shown) were performed with duplicate dot-blot filters hybridized to known single-copy gene probes. These control experiments confirmed the uniformity of the DNA mass loads used in MT blot analyses.

(iii) Southern blot analysis. Methods for quantitative Southern blot analysis have been described previously (4) and were used without modification.

Measurement of MT mRNA concentration. Plasmid DNA or cytoplasmic poly(A)$^+$ RNA was isolated as described above and mixed with either MT-1- or MT-2-specific single-stranded DNA tracers in 3 to 5 μl of 1.5 M NaCl–10 mM Tris hydrochloride (pH 7.5)–2 mM tetrasodium EDTA–0.2% SDS. In paired controls, poly(A)$^+$ RNA or plasmid DNA was replaced with water. The samples were then melted at 100°C for 2 min and incubated at 72°C to the indicated equivalent R_0t, and the fraction of MT tracer present in double-stranded molecules was quantified by measuring resistance to S1 nuclease according to previously published techniques (8). The specificity of the single-stranded DNA probes purified from MT-1 or MT-2 cDNA plasmids for hybridization with either MT-1 or MT-2 DNA sequences was tested under the conditions of the standard cDNA S1 nuclease assay. (It should be emphasized that each of the assays described above for measuring MT gene number quantifies probe sequences colinear with DNA duplexes in addition to probe sequences actually in DNA duplexes. In contrast, S1 nuclease analysis only detects probe sequences in duplexed molecules. Thus, DNA probes that are cross-reactive with the two MT genes by the criterion of hydroxylapatite chromatography or blot analysis may display little or no cross-reactivity when assayed by the criterion of resistance to S1 nuclease.) More than 75% of each single-stranded MT tracer was resistant to S1 nuclease after hybridization to excess polyadenylated RNA from zinc-induced Cd^{r}200T1 cells (e.g., see Fig. 2), but only approximately 5% of each tracer was resistant to S1 nuclease when poly(A)$^+$ RNA was omitted from the reaction mixture (data not shown). In reactions between each tracer and restricted MT-1 or MT-2 plasmid DNAs, at least four to six times more of each MT tracer was reactive with homologous MT DNA than with heterologous MT DNA (data not shown). Therefore, the single-stranded MT tracers, in combination with the S1 nuclease assay, discriminates between MT-1 and MT-2 DNA sequences.

RESULTS

Measurements of MT gene number. To determine the extent of inducibility of each MT gene, it was first necessary to measure accurately the copy number of each MT gene in each Cd^r line. Three independent methods were used to measure the number of MT genes in the Cd^r cell lines. First, the rates of reassociation of several MT DNA tracers with the DNAs from each of the Cd^r Chinese hamster cell lines were measured and compared with the rate of reassociation of an internal single-copy fraction DNA tracer. Three different MT DNA tracers were used for these experiments. The first two MT tracers were derived from recombinant MT cDNA plasmids (7) and are complementary to the protein coding regions of MT-2 and MT-1 mRNA (see Materials and Methods). The third MT tracer was purified from cDNA reverse transcribed from abundant, metal-induced mRNA of Cd^r Chinese hamster cells ($cDNA_a$; reference 8). Some of these experiments have been reported previously (see the legend to Table 1), and therefore only a summary of the results is provided herein. Briefly, the equivalent C_0t at which each hybridization reaction is half complete is proportional to the concentration of genomic DNA sequences complementary to each tracer (24). Therefore, the ratio of the equivalent C_0t of the internal single-copy fraction DNA tracer when the hybridization reaction is half complete (which defines the rate of reaction of the least abundant genomic DNA sequences) to that of the MT DNA tracers when hybridization is half complete defines the number of MT genes in each of the genomes of the Cd^r cells. The results obtained with these three MT tracers were very consistent (Table 1). In combination, the hybridization kinetic data indicate that the MT genes in Cd^{r}2C10 are present at approximately single-copy DNA levels and are amplified in Cd^{r}20F4, Cd^{r}30F9, and Cd^{r}200T1 to approximately 7, 3, and 12 times the Cd^{r}2C10 value, respectively.

The second measurement of MT gene number was obtained by dot-blot hybridization of DNA from each Cd^r Chinese hamster cell to the 160-NT *Bam*HI-*Hin*fI fragment of pCHMT-2 that is complementary to the protein coding regions of both MT-2 and MT-1 mRNAs (7). In contrast to the first method, in which gene number is calculated from the rate of DNA reassociation, in dot-blot analysis, gene number is calculated from the mass of radioactive complementary DNA sequence hybridized by defined samples of immobilized genomic DNA. The results of such an experiment are shown in Fig. 1. The degrees of MT gene amplification estimated from this experiment are very similiar to the MT gene copy numbers calculated from the rates of

TABLE 1. MT gene amplification in Cd^r Chinese hamster cells

Cell line	Minimum no. of MT-1 and MT-2 gene pairs per haploid genome determined by[a]:				
	Solution hybridization kinetics expt no.			Dot-blot hybridization[b]	Southern blot hybridization[c]
	1[b,c]	2[d]	3[e]		
Cd^{r}2C10	1	1	1	1	1
Cd^{r}20F4	7	4–6	10	4–8	10
Cd^{r}30F9	3	2–3	4	2–3	3–5
Cd^{r}200T1	12			4–8	10–20

[a] Since MT-1 and MT-2 genes are amplified coordinately (see the text), these data represent the minimum number of pairs of MT-1 and MT-2 genes.
[b] Data were obtained with the 160-NT *Bam*HI-*Hin*fI fragment of plasmid pCHMT-2.
[c] Data previously reported in reference 4.
[d] Data were obtained with the 63-NT *Hpa*II-*Alu*I fragment of plasmid pCHMT-2.
[e] Data were obtained with cDNA complementary to Cd^{2+}-induced abundant RNA and reported previously in reference 23.

reassociation of the MT DNA tracers with genomic DNA (Table 1).

The MT probes used above cross-hybridize equally with both MT-1 and MT-2 DNA sequences under the conditions used in the reassociation kinetic measurements and dot-blot analyses (4, 7). Therefore, it cannot be determined from these data alone whether it is the MT-1 gene, the MT-2 gene, or both MT genes that are amplified. To address this question, the third measurement of MT gene number was obtained by quantitative Southern blot hybridization of gel-resolved DNAs from each of the Cd^r cells with DNA tracers complementary to one, the other, or both MT DNAs (i.e., MT-1 and MT-2). It has been demonstrated previously that restriction fragments detected by MT-1- and MT-2-specific probes each hybridize approximately equal amounts of *Bam*HI-*Hin*fI MT tracer (4). This was true of each Cd^r cell line examined. This indicates that amplification of MT-1 and MT-2 genes is coordinate. This latter finding is not surprising, since MT-1 and MT-2 genes have been shown to be closely linked in both mouse (3, 20) and Chinese hamster genomes (4, 22). The results of Southern blot hybridization (Table 1) also confirm the first two measurements of MT gene dosage. It is concluded from these several independent studies that the approximate haploid copy numbers of each MT gene in the four Cd^r cell lines are: 1 in Cd^{r}2C10, 7 in Cd^{r}20F4, 3 in Cd^{r}30F9, and 12 in Cd^{r}200T1.

Induction of MT-1 and MT-2 mRNAs in Cd^r cells. Having established the copy numbers of the MT-1 and MT-2 genes in each Cd^r line, I next compared the relative inducibilities of these genes. Cytoplasmic poly(A)$^+$ RNA was extracted from maximally zinc-induced cells and the kinetics of hybridization of each RNA with MT-1- or MT-2-specific DNA tracers were measured in parallel (Fig. 2). In selected experiments, the reaction kinetics of an internal standard of $cDNA_a$, i.e., the [^{3}H]cDNA complementary to zinc-induced, abundant poly(A)$^+$ RNA (also used above to measure MT gene number) was also measured (data not shown). The equivalent R_0t at which hybridization was half complete ($ER_0t_{1/2}$) of each reaction was then used to calculate the relative concentration of the reacting RNA species as described above for genomic DNA (Table 2). The zinc-induced MT-1 mRNA concentrations in the cell lines containing amplified MT genes, i.e., lines Cd^{r}20F4, Cd^{r}30F9, and Cd^{r}200T1, were determined to be equal to 1, 3, and 15 times, respectively, that measured in Cd^{r}2C10, the line in which the MT genes were not demonstrably amplified. Similiarly, the comparable MT-2 mRNA concentrations were equal to 1, 3, and 14 times, respectively, that measured in zinc-induced Cd^{r}2C10. Virtually identical results were also obtained with Cd^{2+}-induced RNAs from the Cd^r cell lines (data not shown). Therefore, the data in Tables 1 and 2 collectively demonstrate that zinc-induced concentrations of MT-1 and MT-2 mRNAs are closely correlated with MT gene copy numbers in Cd^{r}2C10, Cd^{r}30F9, and Cd^{r}200T1. In contrast, whereas Cd^{r}2C10 and Cd^{r}20F4 cells differ by a factor of approximately 7 in their respective MT gene dosages, each induces the same amount of MT mRNA in the presence of 100 μM $ZnCl_2$. Therefore, MT-1 and MT-2 mRNA concentrations do not correlate with MT gene number in Cd^{r}20F4.

Independent verification of these results was obtained by using protein pulse-labeling to estimate the relative amounts of MT mRNA associated with cellular polysomes at various times after the addition of 100 μM $ZnCl_2$ to each Cd^r cell line. There was little, if any, difference between the amounts of pulse-labeled MT made by Cd^{r}2C10 and Cd^{r}20F4 cells at any time examined (Table 3). At the times when each cell line expressed its largest fraction of pulse-labeled MT, the levels in Cd^{r}2C10 and Cd^{r}20F4 were approximately 2 to 3 times lower than that measured in Cd^{r}30F9 and 10 times lower than that measured in Cd^{r}200T1. These results are in excellent agreement with the relative MT mRNA levels measured independently by RNA-DNA hybridization (Fig. 2). Taken together, these data indicate either that the average MT gene in Cd^{r}20F4 is induced only 1/6 to 1/7 as efficiently as its counterpart in any of the other cell lines, or that only 1/6 to 1/7 of the MT genes in Cd^{r}20F4 are inducible.

From the data in Table 2, it was also determined whether the induction of MT-1 and MT-2 mRNAs is coordinate or independent in each of the Cd^r cell lines. Again calculating from the $ER_0t_{1/2}$ of the MT-1 and MT-2 hybridization reactions, the apparent concentration of MT-1 mRNA is five to six times greater than that of MT-2 mRNA in each of the zinc-induced (and cadmium-induced) Cd^r cell lines. Thus,

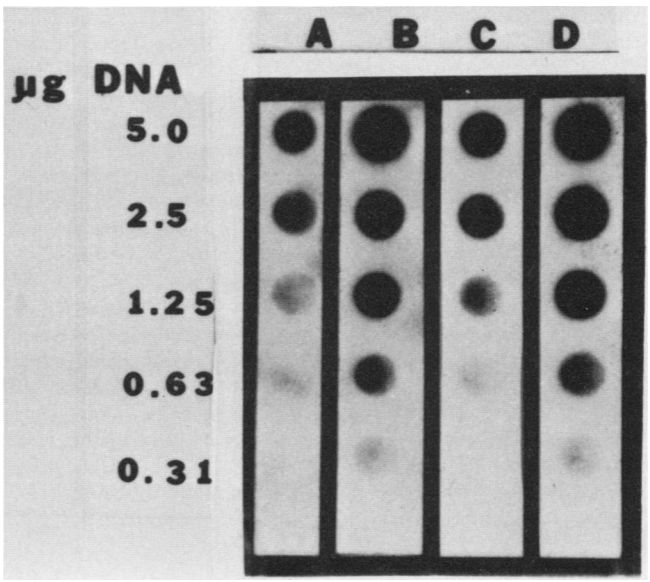

FIG. 1. DNA dot-blot analysis of Cd^r cell DNAs. The indicated amount of high-molecular-weight DNA from each Cd^r cell line was blotted and hybridized with the 160-NT *Bam*HI-*Hin*fI probe as described in Materials and Methods. Lanes: A, Cd^{r}2C10; B, Cd^{r}20F4; C, Cd^{r}30F9; D, Cd^{r}200T1.

whereas the amount of MT mRNA made by each Cd^r cell line differed, the ratio of MT-1 to MT-2 mRNAs was constant.

Expression of MT-2 mRNA in uninduced Cd^r cells. Inability to induce MT expression in proportion to Cd^{r}20F4 MT gene number could reflect defects in either the *trans*-acting factors involved in the metal induction process or the regulatory elements of the MT genes themselves. Therefore, initial experiments were performed to determine the level of expression of the amplified MT gene in uninduced cells. Poly(A)$^+$ RNA was purified from uninduced Cd^{r}2C10, Cd^{r}20F4, and Cd^{r}200T1 cells and mixed with the MT-2 tracer, and the kinetics of hybridization were measured as described above (Fig. 2C). The results of these experiments (Table 2) indicate that the concentration of MT-2 in uninduced Cd^{r}20F4 cells is equal to that in uninduced Cd^{r}2C10 cells and approximately 1/10 of that in uninduced Cd^{r}200T1 cells. Thus, in combination with the gene dosage measurements in Table 1, these results indicate that the level of constitutive MT-2 expression is also closely correlated with MT gene dosage in Cd^{r}2C10 and Cd^{r}200T1 cells but is not correlated with MT gene dosage in Cd^{r}20F4 cells. As in the case of induced MT-2 expression, the equivalent of only 1/6 to 1/7 of the Cd^{r}20F4 genes express MT-2 mRNA constitutively. This suggests that the defect in Cd^{r}20F4 involves DNA sequences that are required for transcription rather than those that are only involved in induction.

From these data, it was also possible to determine whether the fraction of the Cd^{r}20F4 MT genes that was expressed constitutively was induced to normal levels by metal. The ratio of the $ER_0t_{1/2}$ of metal-induced and uninduced RNA reactions with MT-2 tracer was computed for each cell line (Table 2). In each of the three cell lines that were examined, metal induction increased the MT-2 mRNA concentration 90- to 150-fold. This result implies that the inability of a subset of Cd^{r}20F4 MT genes both to induce and to express constitutively the remainder of the MT genes most likely results from alterations in a fraction of the amplified MT genes themselves.

TABLE 2. Hybridization of MT DNA with Cd^r cell RNA

Cell line	Induced MT-1 $ER_0t_{1/2}$[a]	Relative MT-1 mRNA level[b]	Induced MT-2 $ER_0t_{1/2}$[a]	Relative MT-2 mRNA level[b]	Uninduced MT-2 $ER_0t_{1/2}$[a]	Relative induction of MT-2 mRNA (fold)[c]
Cd^{r}2C10	1.0	1	5.5	1	500	90
Cd^{r}20F4	1.0	1	5.5	1	500	90
Cd^{r}30F9	0.35	3	2.0	3		
Cd^{r}200T1	0.065	14	0.4	15	60	150

[a] All data from Fig. 2.
[b] Relative MT mRNA levels were computed from the ratio of the $ER_0t_{1/2}$ of each cell line to the $ER_0t_{1/2}$ of Cd^{r}2C10.
[c] Relative inductions of MT-2 mRNA were computed from the ratio of the $ER_0t_{1/2}$ obtained with induced and uninduced cell RNA.

DISCUSSION

Coordinate induction of MT-1 and MT-2. The data reported here for Chinese hamster cells, in combination with those reported recently for mouse cells (25), convincingly demonstrate that the induction of rodent MT-1 and MT-2 genes is coordinate. In concert, these studies demonstrate

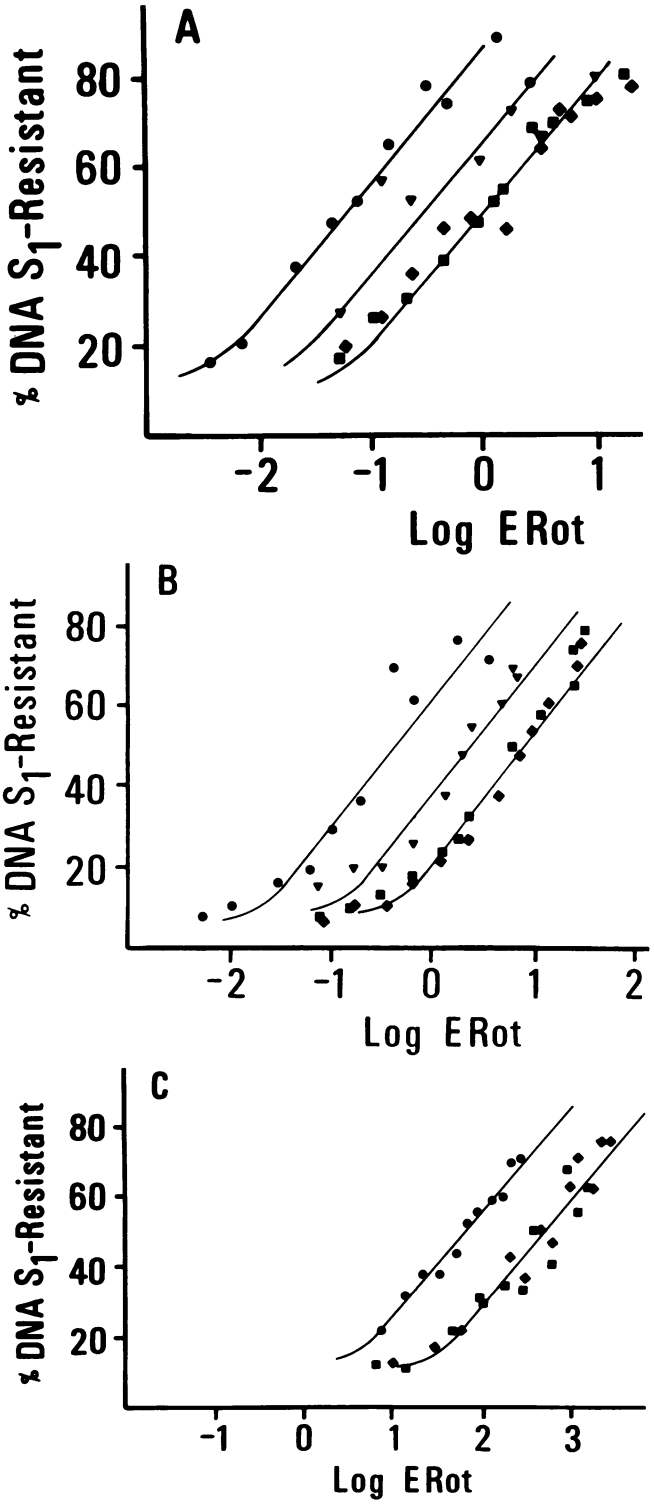

FIG. 2. Zinc induction of MT mRNAs in Cd^r CHO cells. Zinc-induced (A and B) and uninduced (C) poly(A)$^+$ RNA from each cell line was mixed with either the MT-1 (A)- or MT-2 (B,C)-specific DNA tracer, melted, and hybridized to the specified equivalent R_0t, and the fraction of the tracer present in double-stranded molecules was assayed by resistance to S1 nuclease. Symbols: ●, Cd^{r}200T1; ▼, Cd^{r}30F9; ■, Cd^{r}20F4; ◆, Cd^{r}2C10. There was no significant tracer self-reassociation over the 360-min time course of these experiments when poly(A)$^+$ RNA was omitted from the reactions (data not shown).

TABLE 3. Pulse-labeled MT as a fraction of total protein synthesis

Time (h) after adding $ZnCl_2$	Ratio of MT to non-MT protein in[a]:			
	Cd^r2C10	Cd^r20F4	Cd^r30F9	Cd^r200T1
0	0	0	0	0.6
2	0.18	0.16	0.29	3.4
4	0.17	0.23	0.42	2.2
6	0.18	0.20	0.75	2.1
8	0.35	0.32	0.47	ND
10	0.43	0.21	0.43	2.5
17	0.26	0.30	0.44	2.4
25	0.17	0.24	0.22	2.0
Maximum fraction	0.43	0.32	0.75	3.4

[a] Samples of cells were harvested at the specified times, pulse-labeled for 30 min with [^{35}S]cysteine, and fractionated into nuclear and cytoplasmic fractions. The cytoplasmic proteins were resolved by G-75 Sephadex chromatography, and the ratio of MT to non-MT protein was computed as described by Hildebrand et al. (10, 11). ND, Not determined.

that coordination of MT-1 and MT-2 induction is independent of MT gene amplification, proximal chromosome translocation, the extent of inducibility of the MT gene pair, and the nature of the inducer, (i.e., zinc, copper, cadmium, or glucocorticoids). This supports the conclusion that induction of both MT genes is mediated by a common regulatory factor. This could be either a *trans*-acting substance that interacts equivalently with the independent regulatory elements of the MT-1 and MT-2 genes (9, 16, 21, 25), a single *cis*-acting DNA element that affects the transcription of both MT genes coordinately, or both.

Insight into the factors affecting coordinate induction of MT genes is provided by the present study of Cd^r20F4. These cells carry a coordinately amplified domain of MT-1 and MT-2 genes which (on average) is inactive in both constitutive and metal-induced MT expression. Since the subset of MT genes that is active in constitutive MT expression can be induced by metal to the same relative level of expression that occurs in normal cells, it is likely that the factors necessary for normal metal induction are operative in Cd^r20F4 (none of the Cd^r lines are inducible by glucocorticoids). This, in turn, suggests that the alteration responsible for reduced expression is within the MT genes themselves. This could be due to changes in either the transcribed or regulatory portions of the genes resulting from MT gene amplification, chromosome translocation of the Cd^r20F4 MT gene domain, or both.

The organization of the transcribed elements of the amplified Cd^r20F4 MT genes has been investigated by Crawford et al. (4). Restriction fragments that are complementary to MT-1 and MT-2 mRNAs are identical in DNAs from Cd^r20F4, Cd^r2C10, Cd^r200T1, and Cd^r30F9 cells. In other preliminary experiments, these same authors have detected no difference in the restriction fragments complementary to MT-1 and MT-2 mRNAs that were produced with methylation-sensitive and insensitive isoschizmers (B. D. Crawford, personal communication). Since there is no evidence that the transcribed portions of the amplified Cd^r20F4 genes are either aberrantly arranged or hypermethylated, the affected sites may be located in DNA sequences that are not identified by the MT-1 and MT-2 cDNA probes, e.g., a *cis*-acting regulatory element. If the regulatory element controlling metal induction of each MT gene is independent, then two lesions, i.e., one in each MT gene, would be required to inactivate MT-1 and MT-2 expression coordinately. However, if coordinate induction of the two MT genes is mediated by a single *cis*-acting element, then only one lesion would be necessary to inactivate both genes coordinately. Simplicity favors the latter alternative. However, it will be necessary to examine the organization and methylation of the DNA sequences proximal to the sequences complementary to MT cDNA in amplified Cd^r20F4 MT genes before this conclusion can be properly evaluated.

Differential expression of MT-1 and MT-2. The data herein also suggest that each MT gene is subject to independent regulation. This conclusion is based on two different lines of evidence, which each indicate that the MT-1 and MT-2 genes are expressed at different relative levels in uninduced and metal-induced Cd^r cells and, therefore, that metal induction has a different quantitative effect on the expression of each MT gene. The first line of evidence is based on the difference in the extent of MT gene induction measured with the MT-2 probe (Table 2) and that determined previously with a cDNA probe that is complementary to both MT-1 and MT-2 mRNAs ($cDNA_a$). The difference in the $ER_0t_{1/2}$ of the reactions of the MT-2 tracer with metal-induced and uninduced Cd^r2C10, Cd^r20F4, and Cd^r200T1 cell RNAs measured by me indicates that metal induction increases the concentration of MT-2 mRNA 90- to 150-fold (Table 2). In contrast, previous studies of Griffith et al. (8) and Walters et al. (23) indicated that metal induction increases the concentration of the RNA sequences that react with $cDNA_a$ at least 2,000-fold. Walters et al. found that, in uninduced cells, $cDNA_a$ reacted with two different classes of RNA, whose relative abundances differed by a factor of approximately 100. Nearly two-thirds of the $cDNA_a$ tracer reacted with the less abundant RNA class, while about one-third reacted with the more abundant RNA class. In contrast, in maximally metal-induced Cd^r cells, all of the $cDNA_a$ tracer reacted with a single RNA class, whose concentration was about 100 times greater than that of the more abundant class of complementary RNA in uninduced cells. This latter finding agrees well with the results of the present study, which also indicates that the concentrations of MT-1 and MT-2 mRNAs are similiar in induced cells (i.e., about a 2- to 6-fold difference). If it is assumed that the more abundant RNA class in uninduced cells contains MT-2 mRNA (a conclusion based on the fact that one-third of the $cDNA_a$ reacts both with this RNA class and with cloned MT-2 cDNA), then the difference in the concentrations of this RNA class between metal-induced and uninduced cells is approximately 100-fold. This estimate is in good agreement with the 90- to 150-fold metal-induced increase in MT-2 mRNA measured by me. In turn, if the less abundant RNA class in uninduced cells contains MT-1 mRNA (a conclusion based on the fact that two-thirds of the $cDNA_a$ reacts both with this RNA class and with cloned MT-1 cDNA), these data would then mean that the concentration of MT-1 mRNA is increased approximately 10^4-fold by metal induction. This agrees well with the greater than 2,000-fold increase in the concentration of mRNA complementary to the majority of $cDNA_a$ that is induced by metal (23).

An independent, albeit less quantitative, demonstration of the differential induction of MT-1 and MT-2 mRNAs is provided by the data of Crawford et al. (4). These authors compared MT-1 and MT-2 protein synthesis in induced and uninduced Cd^r cells. They found that uninduced Cd^r200T1 cells expressed MT-2 but not MT-1, whereas both MT species were expressed in metal-induced Cd^r200T1 cells. The data in the present study demonstrate that the constitutive concentration of MT-2 in uninduced Cd^r cells is correlated with MT gene dosage, except in Cd^r20F4 (Tables 1, 2,

and 3). Therefore, the failure of Crawford et al. to detect MT-2 synthesis in the other uninduced Cd^r lines is likely due to their lower MT gene dosages and proportionately lower levels of MT-2 mRNA available for translation. Thus, these two independent results together lead to the provisional conclusions that zinc induction causes a much greater increase in the concentration of MT-1 than MT-2 mRNA and that the regulation of the MT-1 and MT-2 genes is noncoordinate in uninduced cells.

An important question that is posed by this observation is whether MT-2 is always expressed differentially in uninduced cells or if there are comparable instances in which MT-1 mRNA is preferentially expressed. As pointed out in the Introduction, the regulatory sequences proximal to the human MT-1 and MT-2 promoters are not functionally equivalent. Hence, the differential regulation of the two MT genes in uninduced rodent cells may also be due to differences in the intrinsic activities of these sequences. On the other hand, Griffith et al. (7) have shown that the differences in the MT-1 and MT-2 mRNA sequences are evolutionarily conserved. This could indicate that the two MT isoproteins each have a different function. In this light, the differential expression of the two MT species may be actively regulated and might be related to differences in the function of each MT. For example, Karin et al. (12) have proposed that MT-2 may normally function in the homeostasis of essential trace metals, whereas MT-1 may be more important in stress response(s). Friedman et al. (6) have also reported that human MT-2 is induced by interferons. Comparing the levels of MT-1 and MT-2 expression in various uninduced cells will be an important step in evaluating the cause and significance of this differential MT expression.

Although the data discussed above suggest that constitutive and metal-induced expression of paired MT genes requires a single *cis*-acting regulatory element, they also suggest that the two MT genes are regulated independently. This implies that at least two levels of control regulate MT gene expression.

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